Preparative chromatography of myosin on hydroxyapatite

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In an earlier paper[1], we described a procedure for the analytical chromatography of myosin on hydroxyapatite that separates it from low-molecular-weight contaminants and from aggregated myosin. The procedure could not be used for preparative purposes because about two-thirds of the ATPase activity was lost, and the specific activity of the effluent was low. We have since found, and herewith report, simple modifications that convert the analytical procedure into a preparative method and yield an additional protein fraction. The loss of activity is prevented by adding mercaptoethanol to the protein sample and to all buffers, including the buffer with which the resin is initially equilibrated. By introduction of an additional elution step (B1) the fraction previously eluted with 0.23 *M* phosphate is resolved into two. Elution with 1 *M* phosphate has been eliminated because no additional protein is eluted by buffers with phosphate concentrations greater than 0.4 *M*. We have thus arrived at a four-step elution program, with the phosphate concentration in the eluant varying from 0.16 to 0.40 *M*. The concentrations of potassium chloride and mercaptoethanol are held constant, as is the pH. The first step elutes a fraction of low molecular weight with no ATPase activity. The other three fractions all have enzymatic activity.

Various media have been used for the chromatographic fractionation of preparations of myosin, including DEAE-cellulose[2], DEAE-Sephadex[3], and agarose[4]. A major advantage of the procedure reported here, using hydroxyapatite, is that the four fractions are eluted successively by stepwise increase of the phosphate concentration of the eluant, thus permitting control of the resolution of fractions from one another. The resolution achieved with hydroxyapatite is consequently superior to that obtained with the other media.

MATERIALS AND METHODS

The hydroxyapatite used was Bio-Gel HT**, produced by Bio-Rad Labs., Richmond, Calif., U.S.A.

Myosin was prepared from the *Longissimus dorsi* muscle of the rabbit as

previously described[1], with the following exceptions: (1) β-mercaptoethanol, at a concentration of 10 m*M*, was present in all reagents used in making the protein preparation, (2) the treatment with ribonuclease was omitted, and (3) deaminase was removed prior to the last (third) precipitation by adsorption onto cellulose phosphate[5].

Chromatography on hydroxyapatite was carried out for the most part as previously described[1]. The major departure in the present procedure is in the elution buffers; these had the compositions given in Table I. Pharmacia columns with jackets maintained at 2–5° were used. The fraction collector and the cell compartments of the monitoring spectrophotometers were also held at this temperature. Other conditions are given in Table II.

The column effluent was monitored continuously for the levels of protein and enzyme with a monitoring system designed for this purpose. The monitoring period was approximately 6.5 min per cycle. Protein concentration was calculated from the transmittances of the column effluent at 279 and 330 nm, the latter being used to correct for light scattering (ref. 1, eqn. 2). These measurements were made with a Beckman DB spectrophotometer. ATPase activity was determined by measurement of ADP production[6] in a continuous procedure to be described elsewhere. The required absorbance measurements were made with a Gilford Model 2000 Multiple Sample Absorbance Recorder system. The data, consisting of the voltage outputs of the two spectrophotometers, were collected on tape and processed with an IBM 1130 computer.

TABLE I

COMPOSITION OF ELUTION BUFFERS

Elution buffer	*Concentration of components*			*pH at 5°*
	KCl (M)	*Phosphate* (M)*	*Mercaptoethanol (mM)*	
A	0.40	0.16	10	6.8
B1	0.40	0.20	10	6.8
B2	0.40	0.23	10	6.8
C	0.40	0.40	10	6.8

* Potassium salts.

TABLE II

CONDITIONS USED FOR CHROMATOGRAPHY ON HYDROXYAPATITE

	Column size	
	Small	*Large*
Column diameter (cm)	1.8	2.54
Column length (cm)	15–18	35
Protein in sample (mg)	45–50	200*
Volume of sample (ml)	3–4	14–17
Volume of each eluent (ml)	75	250**
Flow-rate (ml/h)	3	20

* The upper limit has not been established, but is likely to be greater than 375 mg[1].

** Volumes up to 500 ml were used in some instances. When this was done, no effect was observed on the elution volumes for subsequent eluents.

The results were presented in the form of chromatograms of protein concentration and enzymatic activity as functions of elution volume, these being drawn by a plotter under control of the computer.

RESULTS AND DISCUSSION

A typical chromatogram is shown in Fig. 1. With a fresh preparation of myosin, about two thirds of the protein applied to the column are recovered in the effluent,

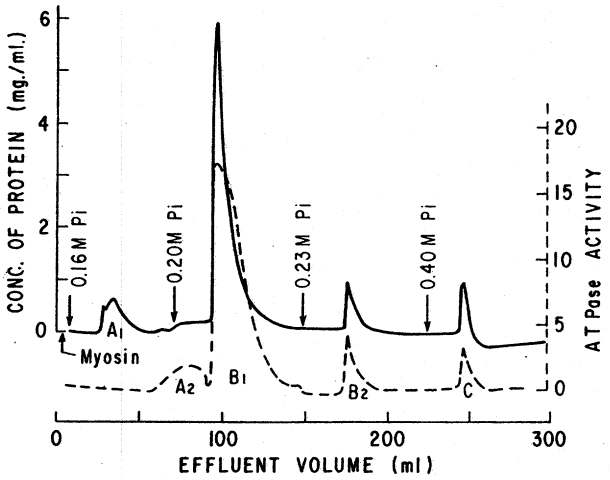

Fig. 1. Elution of protein and ATPase activity from hydroxyapatite. The numbers adjacent to the arrows give the phosphate concentrations in the elution buffers. The composition of the buffers is given in Table I. ———, Protein concentration; ---------, ATPase activity, in arbitrary units.

and about three quarters of this appear in peak B1. Close to one half of the applied protein thus appears in the major fraction, the fraction eluted by the buffer containing 0.20 *M* phosphate. The figure shows that a small amount of protein with activity (A2) emerged just prior to the major peak. This feature is variable. The peak is frequently absent, and, when present, the volume of buffer A required to elute it is variable. The first peak (A1) has no ATPase activity and is dialyzable. The specific activity of the major peak varies from approximate equality to that of the preparation applied to the column to a value about twice that of the sample applied.

Fractions B2 and C both have activity, and are virtually indistinguishable from the major fraction, B1, when subjected to gel electrophoresis in the presence of sodium dodecyl sulfate. In our earlier procedure[1], eluent B1 (Table I) was not used. Fractions B1 and B2 were therefore eluted together, as fraction B. On the basis of the ultracentrifugal patterns of fractions B and C and the behavior of these fractions on rechromatography, as well as other supporting evidence previously discussed[1], it was concluded that C is an aggregated form of myosin, it being implied that B consists of the unaggregated protein. In view of this conclusion and the similarity of fractions B1, B2, and C in enzymatic activity and in their behavior on electrophoresis, it appears reasonable to assume that B2 and C are both aggregated forms of myosin. The

difference between B2 and C may reside in the extent of aggregation. Because on aging of a preparation of myosin the amount of protein bound irreversibly to hydroxyapatite increases[1], it may be assumed that binding increases with the extent of aggregation. On this basis, it is to be expected that fraction C is more highly aggregated than B2, B1 being unaggregated.

CONCLUSIONS

The previously published procedure[1] for chromatography of myosin on hydroxyapatite has been modified to make it useful for preparative as well as analytical work. The whole procedure is carried out at a pH near neutrality, at high ionic strength, and in the presence of substantial levels of orthophosphate. Stepwise increase of phosphate concentration permits simple control of the resolution of fractions; the resolution achieved is better than that generally seen in myosin chromatography by other means[2-4]. The method achieves removal from myosin of a contaminant of low molecular weight as well as complete separation of two additional fractions which appear to be myosin in different states of aggregation.

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